

Cell adhesion molecules in stromal corneal dystrophies

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Summary. The aim of the present study was to investigate the expression pattern of different cell adhesion molecules in corneal stromal dystrophies. Fifteen corneal buttons from patients diagnosed with three different types of stromal corneal dystrophies and healthy corneas were investigated. Paraffin embedded sections were stained immunohistochemically with monoclonal antibodies against human intercellular adhesion molecule-1 (ICAM-1), endothelial selectin (E-selectin) and endothelial cadherin (E-cadherin) using the avidin-biotin-peroxidase-complex technique. The sections were compared to normal eye bank controls.

In corneas from granular dystrophy patients ICAM-1 was expressed focally in epithelial cells and in keratocytes, and expressed diffusely in endothelial cells. In corneas from macular dystrophy patients diffuse epithelial staining was observed and the stromal and endothelial expression was found to be similar to that of granular dystrophy. In lattice dystrophy, only the epithelial cells and endothelium were intensively positive for ICAM-1. E-selectin was not present on any layer of the corneal specimens. E-cadherin was observed only in the epithelium of all three types of corneal dystrophies. Normal corneas did not express any of the investigated adhesion molecules.

We found different expression patterns of adhesion molecules in corneas from stromal dystrophies. Our results suggest that adhesion molecules may be involved in the pathogenesis of corneal stromal dystrophies.

Key words: Stromal corneal dystrophy, Cell adhesion molecule, Intercellular adhesion molecule-1, E-selectin, E-cadherin, Immunohistochemistry

Introduction

Cell adhesion molecules, together with cell junctions, are responsible for the establishment of interactions between cells and extracellular matrix. During development, the turnover of tissues and alteration of the expression pattern of these glycoprotein molecules are dynamically regulated (Gumbiner, 1996). Four large families of adhesion molecules have been defined: the integrin, the selectin, the immunoglobulin and the cadherin family (Dustin and Springer, 1991).

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin family. Endothelial selectin (E-selectin), previously known as endothelial leukocyte adhesion molecule-1 (ELAM-1), is a surface glycoprotein and its lectin-like domain can recognize and bind to carbohydrates. E-cadherin is a calcium-dependent cell adhesion molecule, which is a developmental cell surface protein responsible for maintaining the structural integrity of the epithelial monolayer. Although cadherins are often thought to mediate stable cell interactions, it has been shown that cadherins play important roles in mediating cell rearrangements and cell migration (Marsden and DeSimone, 2003). Corneal dystrophies are predominantly inherited conditions manifested by non-inflammatory opacities in the cornea. Among these disorders, stromal dystrophies are one of the most common indications for penetrating keratoplasty (Cursiefen et al., 1998). In granular dystrophy, corneal opacities are confined to the anterior layers of the stroma and usually there is a typical snowflake appearance in the center. In macular dystrophy, multiple grayish white opacities involve the stroma and the corneal periphery, and the stroma between the opacities is diffusely cloudy. In lattice dystrophy, lattice-like opacities are present in the anterior part of the cornea (Kanski, 2003).

The potential genetic basis of corneal dystrophies is widely examined. Mutations in a sulphotransferase gene was observed (Akama et al., 2000; Iida-Hasegawa et al.,

2003) and the potential role of BIGH3/TGFB1 gene was also studied (Munier et al., 2002, Hilton et al., 2007).

The central corneal stroma is mostly composed of collagen I, III, IV, V and VI and extracellular matrix such as glucoseaminoglycans and proteoglycans (Marshall et al., 1991a,b). However, the role of adhesion molecules in cell-cell, cell-collagen and cell-matrix interaction has not been sufficiently investigated. Moreover, their role in the pathogenesis of different corneal dystrophies is still obscure.

The purpose of this study was to investigate the expression pattern of different types of cell adhesion molecules in corneal stromal dystrophies. To the best of our knowledge, this study reports for the first time the examination of expression pattern of ICAM-1, E-selectin and E-cadherin in granular, macular and lattice dystrophy of the corneal stroma.

Materials and methods

Corneal specimens

8 specimens of granular dystrophy type I, 3 specimens of macular dystrophy and 4 samples of lattice dystrophy type I were investigated. The specimens were derived from patients who underwent penetrating keratoplasty. The clinical diagnosis of corneal dystrophies was established by two independent physicians according to the clinical history and slit-lamp appearance. The genetic status was not ascertained and the patients were from different pedigrees.

A random group of age-matched healthy corneas harvested from donors unsuitable for corneal transplantation served as normal controls. These eyes had no sign of previous disease. Shortly, after death - in the case of cadavers, typically within 6 hours - the samples were removed and immediately transferred into 10% neutral formalin fixative and left overnight at 4 °C. To obtain the control corneas the same protocol has been followed as described above. After specimen fixation in 10% neutral formalin, samples were embedded in paraffin and serially sectioned as thick as the experimental groups at 7 µm.

The study protocols were reviewed and approved by the Ethical Committee of Human Experiments of the Medical and Health Science Center, University of Debrecen, Hungary, in accordance with national laws, and were properly carried out in compliance with the University's Guidelines for Human Experimentation.

Histological preparation

The routine method for fixation and wax embedding of the corneal samples has been previously described (Takacs et al., 1999). After fixation and dehydration, tissue samples were embedded in wax at 54°C, and 7 µm thick sections were cut (Leica Instruments, Nussloch, Germany). Paraffin sections were placed on gelatin-coated glass slides and left to dry overnight at 37°C.

Histochemistry

Tissue sections from experimental and control groups were prepared using standard histological techniques. Tissues were fixed in the same way described earlier and embedded in paraffin (Takacs et al., 1999). Before routine immunohistochemical staining, sections were deparaffinized in xylene and hydrated through graded alcohols to water.

Haematoxylin-eosin, periodic acid Schiff, colloidal iron and Congo red stainings have been carried out to determine the type of stromal dystrophies.

All stained slides were washed in distilled water, dehydrated through graded alcohols, cleared in xylene, and mounted in DPX mountant (Fluka Chemie GmbH, Buchs, Switzerland).

Immunohistochemistry

Immunohistochemical studies were performed using 7 µm, formalin-fixed, paraffin-embedded sections. A panel of anti-mouse antibodies was used: ICAM (Batch: 130704, Novocastra, Newcastle, UK), E-selectin (Batch: 138203S, Novocastra, Newcastle, UK) and E-cadherin (Batch: 138506, Novocastra, Newcastle, UK). The avidin-biotin peroxidase complex technique used in this study has been described earlier (Felszeghy et al., 2005). Briefly, after dewaxing, dehydration and blocking the endogenous peroxidase activity, the slides were preincubated in 1% BSA dissolved in PBS (pH: 7.4) for 30 minutes at 37°C in order to prevent non-specific binding of the primary antibodies. Then the sections were incubated overnight at 4°C with the above-mentioned antibodies. The antibodies were used at the following dilutions: ICAM (1:50), E-selectin (1:50) and E-cadherin (1:100). The labeling was visualized by use of peroxidase-conjugated secondary antibodies (Vectastain Elite KIT 1:200, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine (DAB) containing 1 µl to 1 ml 30% H₂O₂. After color development, the sections were washed in distilled water, dehydrated with ethanol, cleared in xylene and mounted in DePeX (BDH Laboratory Supplies, Poole, UK). Control sections were stained in the same way but the primary antibody was omitted and replaced by non-immune IgG (IgG_{2a}; Sigma). No signal was recorded from control sections incubated with non-immune IgG instead of primary antibody. Sections of human skin were used as positive controls.

Analysis of data and image capturing

Light microscopy

An overview of immunostained slides was obtained with light microscopy (Nikon Eclipse E 600, Japan), and images were captured with the same microscope equipped with a digital camera (Spot RT, Germany).

Acquired and presented images are representative of

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all the tissue sections examined during our study. For documentation, images were processed using Adobe PhotoShop software (Version 5.5, Adobe Systems Inc., San Jose, CA). All clinically and histologically negative controls revealed no immunospecific staining, so tissue sections did not show any endogenous peroxidase activity. The intensity of immunopositivity (staining) was graded as mild, moderate and marked expression. Immunostaining studies were performed in duplicate using tissues obtained either from different corneal

dystrophies or normal human corneas as controls. The types of the dystrophies and controls have been described in the preceding paragraphs pertaining to the corneal specimens above. Two researchers determined cell identification and localization for each molecule and grade independently in the studied samples.

Results

The distribution and intensity of expression of

Table 1. The distribution and intensity of immunopositivity of different adhesion molecules in our corneal specimens.

	ICAM-1			E-selectin			E-cadherin		
	Epithelium	Stroma	Endothelium	Epithelium	Stroma	Endothelium	Epithelium	Stroma	Endothelium
Granular dystrophy	++	+	+++	-	-	-	+++	-	-
Macular dystrophy	++/+++	+	+++	-	-	-	+++	-	-
Lattice dystrophy	++	+	+++	-	-	-	++	-	-

-: no expression, +: mild expression; ++: moderate expression; +++: marked expression.

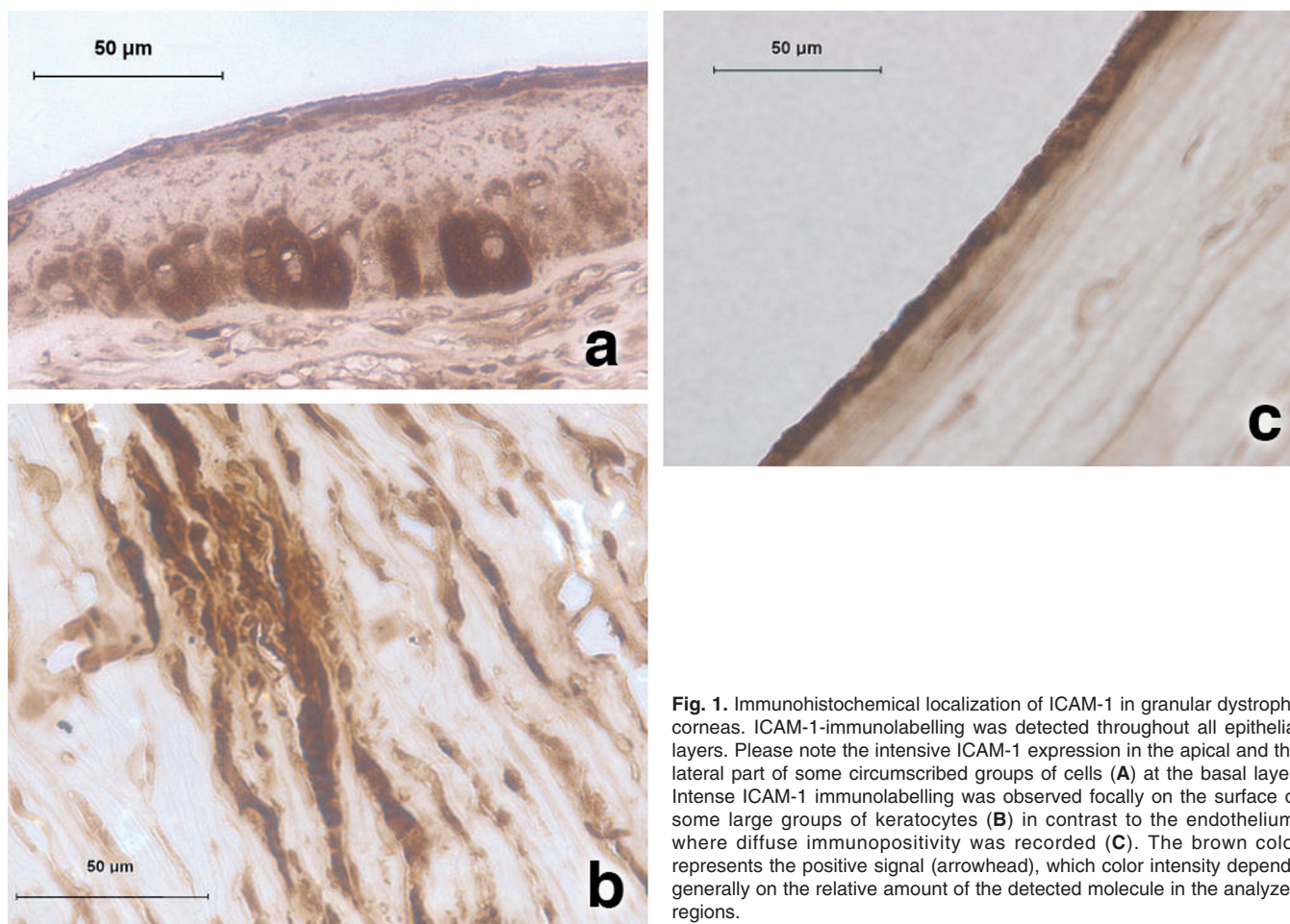


Fig. 1. Immunohistochemical localization of ICAM-1 in granular dystrophy corneas. ICAM-1-immunolabelling was detected throughout all epithelial layers. Please note the intensive ICAM-1 expression in the apical and the lateral part of some circumscribed groups of cells (A) at the basal layer. Intense ICAM-1 immunolabelling was observed focally on the surface of some large groups of keratocytes (B) in contrast to the endothelium, where diffuse immunopositivity was recorded (C). The brown color represents the positive signal (arrowhead), which color intensity depends generally on the relative amount of the detected molecule in the analyzed regions.

different adhesion molecules in our corneal specimens are summarized in Table 1.

Expression of ICAM-1

In corneas from granular dystrophy patients, ICAM-1 was detected in the corneal epithelium, keratocytes and endothelial cells. In epithelial cells, ICAM-1 was present in all epithelial layers, but was intensively marked in the basal cells, typically in the apical and the lateral part of these cells. Interestingly, some circumscribed groups of cells were intensively stained (Fig. 1A). The immunopositivity was increased focally on the surface of some large groups of keratocytes (Fig. 1B). The

immunopositivity was observed only in the endothelium (Fig. 1C).

In macular dystrophy, marked ICAM-1 positivity was observed on epithelial cells (Fig. 2A), except surface epithelial layers. The stromal (Fig. 2B) and endothelial expression was similar to that of granular dystrophy.

In corneas from lattice dystrophy patients, only the basal and the superficial cells of the epithelium were positive; the intermediate layers remained unstained (Fig. 3A). In the stroma, a weak signal was seen on scattered keratocytes (Fig. 3B) and the endothelium showed the same expression of ICAM-1 as in granular or macular dystrophy.

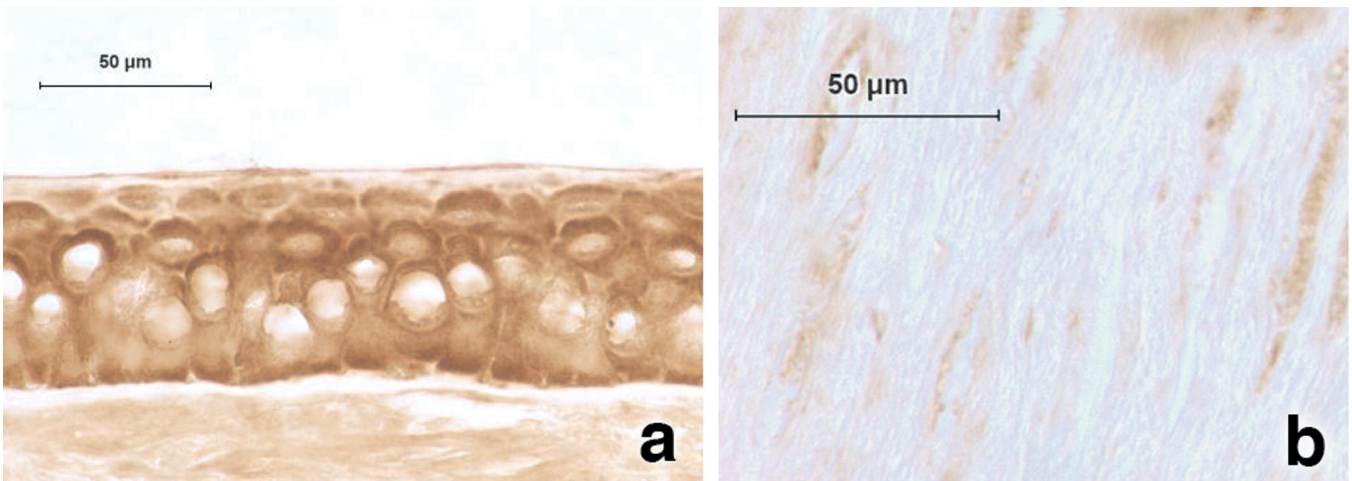


Fig. 2. Immunohistochemical localization of ICAM-1 in corneas from macular dystrophy patients. The immunopositivity was marked on corneal epithelial cells (A), except for superficial layers. Immunopositivity was only focally on the surface of small groups of keratocytes (B).

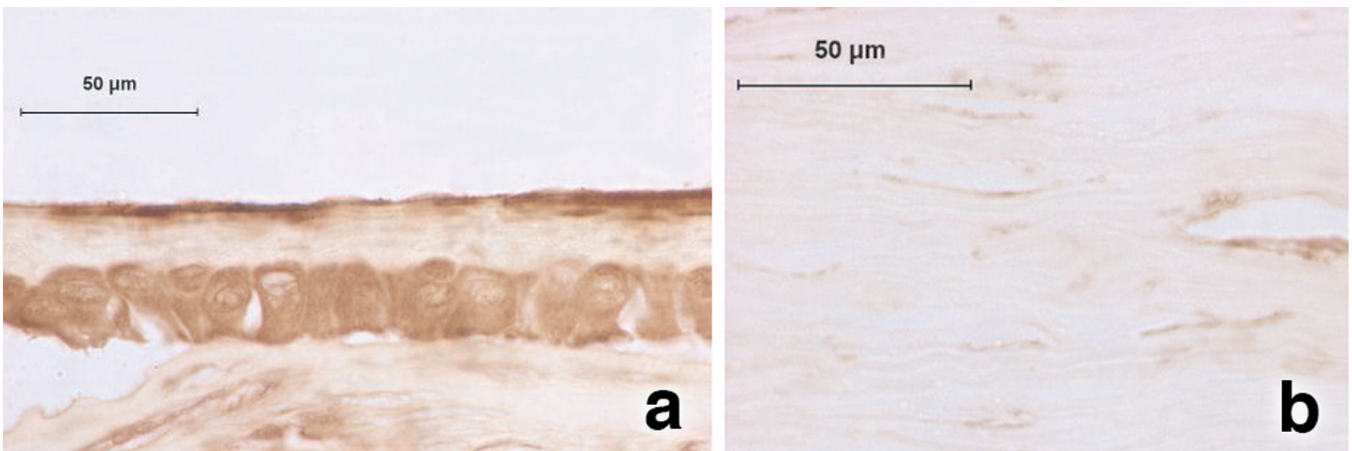


Fig. 3. Immunolabeling for ICAM-1 in corneas from lattice dystrophy patients. Only the basal and the superficial cells of the corneal epithelium expressed ICAM-1; the intermediate layers remained unstained (A). A weak signal was seen on scattered keratocytes (B).

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Expression of E-selectin

None of the three examined dystrophies disclosed any staining in any layers of the cornea for E-selectin.

Expression of E-cadherin

In corneas from granular (Fig. 4A) and from macular (Fig. 5A) dystrophy patients, the border of all cellular layers of the epithelium was positive for E-cadherin. In corneas from lattice dystrophy patients, only the basal layer of the epithelium expressed E-cadherin (Fig. 6A).

The stroma did not show any expression for E-cadherin either in granular (Fig. 4B), macular (Fig. 5B)

or in lattice dystrophies (Fig. 6B). The endothelium was also negative for E-cadherin in these three types of corneal dystrophies.

Normal corneas

The normal, healthy corneas revealed no immunostaining.

Discussion

The present study investigated the distribution of cell adhesion molecules in corneal stromal dystrophies and in normal corneas. Cell adhesion molecules promote

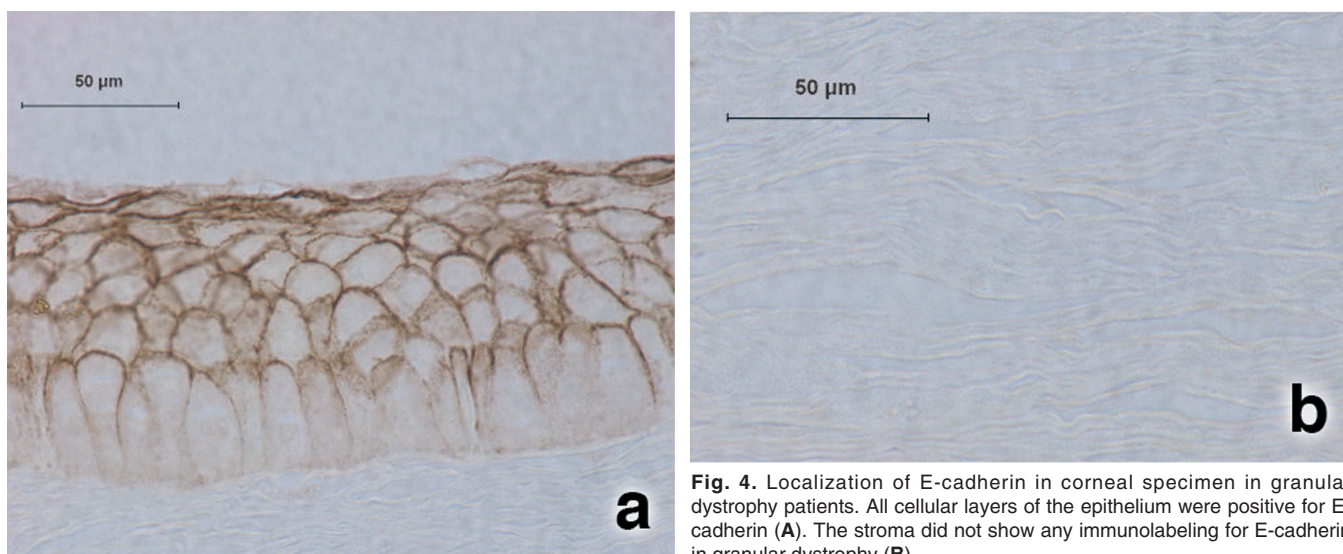


Fig. 4. Localization of E-cadherin in corneal specimen in granular dystrophy patients. All cellular layers of the epithelium were positive for E-cadherin (A). The stroma did not show any immunolabeling for E-cadherin in granular dystrophy (B).

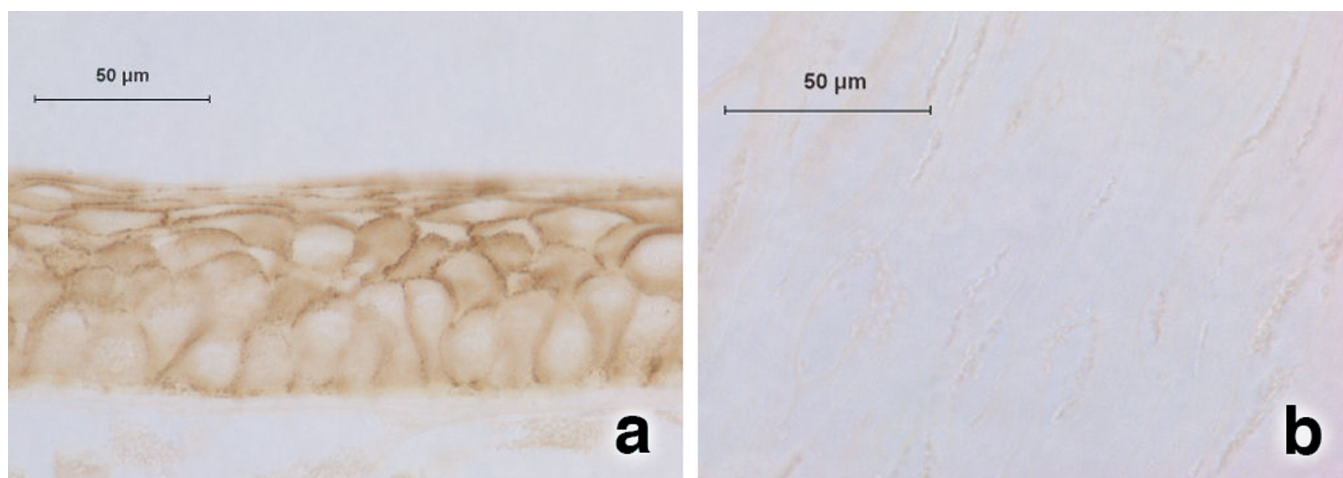


Fig. 5. Immunohistochemical localization of E-cadherin in corneas from macular dystrophy patients (A). The border of all cellular layers of the corneal epithelium expressed E-cadherin. The stroma did not show expression for E-cadherin in corneas from macular dystrophy patients (B).

various types of cell-cell and cell-extracellular matrix interactions. These molecules are surface glycoproteins, which may play an important role in the pathogenesis of different corneal diseases. There are some data available about the pathogenetic role of adhesion molecules in corneal graft failure (Philipp, 1994; Whitcup et al., 1993), in inflamed corneas (Goldberg et al., 1994), in bullous keratopathy (Zhu et al., 1996), and in keratoconus (Philipp et al., 1993a,b). However, the

distribution of cell adhesion molecules in human corneas with different pathologies is still a concern.

Previous studies described the expression of ICAM-1 in keratocytes and cultured endothelial cells at low levels in normal corneas, but its expression increases with inflammatory conditions upregulated by different cytokines, such as in keratitis or chemical corneal burns (Goldberg et al., 1994; Pavilack et al., 1991, 1992). In Pavilack's study, ICAM-1 expression is increased

Table 2. Summary of adhesion molecules in normal and pathologic corneas according to the literature.

Author(s)	Adhesion molecule	Corneal disease	Affected corneal layer
Whitcup et al. (1993)	ICAM-1	Normal	Negative
Elnor et al. (1991)	ICAM-1	Normal	Keratocyte, endothelium
Vorkauf et al. (1995)	ICAM-1	Normal	Keratocyte, endothelium
Elnor et al. (1991)	ICAM-1	Normal	Keratocyte, endothelium
Pavilack et al. (1992)	ICAM-1	Normal	Keratocyte, endothelium
Philipp and Gottinger (1993b)	ICAM-1	Normal and inflamed	Epithelium, keratocyte, endothelium
Foets et al. (1992)	ICAM-1	Normal, diseased	Endothelium
Goldberg et al. (1994)	ICAM-1	Inflammation	Epithelium, keratocyte, endothelium
Vorkauf et al. (1995)	ICAM-1	Inflammation	Epithelium, keratocyte, endothelium
Whitcup et al. (1993)	ICAM-1	Graft rejection	Epithelium, keratocyte, endothelium
Philipp (1994)	ICAM-1	Keratitis, graft rejection	Epithelium, keratocyte, endothelium
Pavilack et al. (1992)	ICAM-1	Cytokine induced keratitis	Epithelium, keratocyte, endothelium
Zhu et al. (1996)	ICAM-1	Bullous keratopathy	Epithelium, keratocyte, endothelium
Philipp and Gottinger (1993b)	ICAM-1	Keratoconus	Keratocyte, endothelium
Vorkauf et al. (1995)	ICAM-1	Keratoconus, endothelial decompensation	Keratocyte, endothelium
Vorkauf et al. (1995)	E-selectin	Normal, keratoconus, bullous keratopathy	Negative
Philipp and Gottinger (1993b)	E-selectin	Normal, keratoconus, bullous keratopathy	Negative
Bevilacqua et al. (1989)	E-selectin	Inflammation	Endothelium
Philipp and Gottinger (1993a)	E-selectin	Inflammation	Endothelium
Philipp and Gottinger (1993a)	E-selectin	Inflammation	Stromal vessel's endothelium
Vorkauf et al. (1995)	E-selectin	Inflammation	Stromal vessel's endothelium
Philipp (1994)	E-selectin	Graft rejection	Stromal vessel's endothelium
Whitcup et al. (1993)	E-selectin	Graft rejection	Keratocyte
Zhu et al. (1996)	E-selectin	Bullous keratopathy	Endothelium
Whitcup et al. (1992)	E-selectin	Endotoxin induced acute uveitis	Endothelium
Whitcup et al. (1992)	E-selectin	Posterior chronic uveitis	Negative

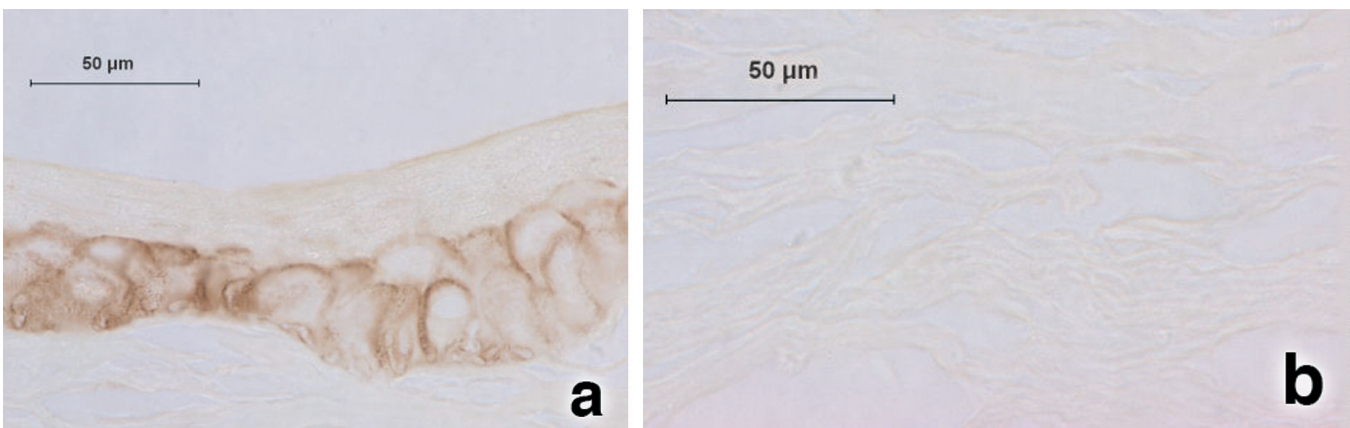


Fig. 6 Immunohistochemical localization of E-cadherin in corneas from lattice dystrophy patients. Only the basal layer of the corneal epithelium expressed E-cadherin (A). The stroma did not show expression for E-cadherin. (B).

significantly on the surface of all types of corneal cells after stimulating with physiologic concentration of human recombinant cytokine, which was most marked in the corneal endothelium (Pavilack et al., 1992).

Others described weak ICAM-1 expression in corneal endothelial cells and in keratocytes in normal corneas (Pavilack et al., 1991, 1992). On the other hand, ICAM-1 was focally expressed predominantly at the basal but all cellular layers of epithelial cell border and cytoplasm (Vorkauf et al., 1995). This observation was very similar to that in our specimens.

There were neither clinical nor histological signs of inflammation in our patients and specimens. However, it is well known that patients with stromal dystrophies often treated for recurrent corneal erosions (Campos et al., 1993; Seitz et al., 2002). Becker et al. (1995) have found increased levels of interleukin-1 β and interleukin-6 in corneas with inflammatory signs. They also found increased levels of the above cytokines in patients with corneal dystrophies; however, the interleukin levels were higher in ulcerating processes. In case of an epithelial injury, cytokin mediators (master regulator: interleukin-1) are released from the damaged tissues and activate apoptosis of keratocytes, corneal fibroblasts and proliferation and migration of the remaining keratocytes (Philipp et al., 1994). We suggest that in our patients, chronic disruption of the irregular epithelium may have led to an increase in the amount of cytokines (interleukin-1, FAS ligand protein, bone morphogenic proteins 2 and 4, bcl2 protein (Mohan et al., 1997, 1998) and ICAM-1 in epithelial cells, keratocytes or both. Our observed results may derive from cytokine release as part of the tissue injury and repair process, resulting from the corneal damage caused by the abnormal deposits which characterize these types of dystrophies.

In our corneal specimens the corneal epithelium showed expressive ICAM-1 positivity in stromal dystrophies and the distribution was different among dystrophies. ICAM positivity was present in all cells of the epithelial layer of granular dystrophy corneas but was intensively marked in the basal cells. In lattice dystrophy, only the basal and superficial cells of the epithelium were immunostained. In contrast, in macular dystrophy, ICAM-1 positivity was rather diffuse. This fact speaks in favor of those findings that granular and lattice dystrophies may derive from the same family of corneal dystrophies (disorders of the chromosome 5 in both dystrophies) and macular dystrophy probably from a separate family (Klintworth, 2003).

E-selectin is expressed on the surface of the vascular endothelium and plays a major role in neutrophil and polymorphonuclear cell adhesion to the cytokine-stimulated endothelial cells (Bevilacqua et al., 1989) in acute inflammation. Whitcup et al. observed increased E-selectin expression in areas of keratic precipitates of corneal endothelium in rats with endotoxin induced acute uveitis (Whitcup et al., 1992). They did not observe any expression of E-selectin in posterior chronic uveitis (Whitcup et al., 1992), so according to them it

seems that E-selectin plays a role only in acute inflammation. This could be the reason for the absence of E-selectin expression in stromal dystrophies without any sign of inflammation in our corneal specimens. On the other hand, others described definite expression of E-selectin in chronic inflammatory corneal diseases (Philipp et al., 1993a). The distribution of adhesion molecules published in the literature is summarized in Table 2.

E-cadherin is a calcium-dependent cell adhesion molecule responsible for maintaining the structural integrity of the epithelial layer. Most interestingly, its expression is similar in the epithelial layer of corneas with granular and macular dystrophy (diffuse staining of cell borders), but in lattice dystrophy only the basal epithelial cells show positivity. To date, there are few data about the expression of E-cadherin in normal or diseased corneas (Scott et al., 1997). In our specimens significant E-cadherin expression was observed in the epithelial layer of all dystrophies.

Based on our findings we conclude that adhesion molecules such as ICAM and E-cadherin may have a pathogenic role in the origin of stromal corneal dystrophies. Further studies need to clarify whether these molecules can help to repair the surface integrity of the diseased corneas caused by the abnormal deposits.

In summary, to our best knowledge, this is the first paper describing the distribution pattern of cell adhesion molecules in corneal stromal dystrophies. We observed diverse, unexpectedly selective, increased expression of cell adhesion molecules in specimens of corneal stromal dystrophies without any sign of inflammation compared to control corneas.

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